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(54) Title: USE OF DECREASING LEVELS OF FUNCTIONAL TRANSIENT RECEPTOR POTENTIAL GENE PRODUCT

(57) Abstract

The invention provides a method of decreasing inflammatory gaps in pulmonary endothelial cells, the method comprising decreasing levels of functional transient receptor potential gene product in the cells. The invention further provides a method of treating or preventing an inflammatory condition in a subject, the method comprising administering to the subject an amount of a compound effective to decrease levels of functional transient receptor potential gene product in the cells of the subject.

## USE OF DECREASING LEVELS OF FUNCTIONAL
## TRANSIENT RECEPTOR POTENTIAL GENE PRODUCT

This application claims priority of U.S. Provisional

5   Patent Application No. 60/093,968, filed July 24, 1998.


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States Government under Grant Nos. HL-56050 and HL-60024
of the National Institutes of Health.  The U.S.
10  Government may have certain rights in this invention.


### FIELD OF THE INVENTION

The subject invention is directed generally to a
method for treating or preventing an inflammatory
15  condition, by decreasing inflammatory gaps in pulmonary
endothelial cells, and more particularly to decreasing
levels of functional transient receptor potential gene
product.


20                 **BACKGROUND OF THE INVENTION**

Throughout this application various publications are
referenced, many in parenthesis.  Full citations for each
of these publications are provided at the end of the
Detailed Description.  The disclosures of each of these
25  publications in their entireties are hereby incorporated
by reference in this application.

Pulmonary endothelial cells are a nonexcitable cell
type in which humoral and neural signaling agents
increase the free cytosolic $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) by
30  inducing $Ca^{2+}$ release from intracellular stores and $Ca^{2+}$
entry across the cell membrane (4,34).  Increased $[Ca^{2+}]_i$
has been implicated in many endothelial-directed vascular
responses including regulation of vascular tone and
permeability (2, 23, 36), angiogenesis (20), and
35  leukocyte trafficking (17).  Activation of $Ca^{2+}$ entry
appears essential for each of these processes, although
many modes of $Ca^{2+}$ entry exist and a specific pathway

regulating endothelial cell shape has yet to be
identified.

It is widely accepted that endothelial cells possess
capacitative, or store-operated, $Ca^{2+}$ entry pathways (8,
5  13, 31, 35, 41, 42). However, specific store-operated
$Ca^{2+}$ channels (SOCs) responsible for $Ca^{2+}$ entry into
nonexcitable cell types are largely unidentified. Recent
cloning and expression of the transient receptor
potential (*trp*) gene product from the *Drosophila*
10 *melanogaster* retina reveal that this product forms a $Ca^{2+}$-
permeant cation channel that mediates $Ca^{2+}$ entry after
intracellular inositol 1,4,5-trisphosphate [Ins(1,4,5)$P_3$]
is generated and $Ca^{2+}$ is liberated from intracellular
stores (11, 15, 25). Six mammalian homologues of
15 *Drosophila* Trp are known (5), and mRNAs for these have
been reported in bovine aortic endothelial cells (12).

Non-cardiogenic pulmonary edema(s) represent a
significant clinical complication that increases patient
morbidity and mortality. Adult respiratory distress
20 syndrome (ARDS) is the most severe form of these diseases
and impacts conservatively 150,000 patients annually.
Non-septic ARDS has an estimated mortality rate of 40-60%
whereas septic ARDS has an estimated mortality rate
exceeding 90%. Clinical management of ARDS patients is
25 supportive, and unfortunately not a single pharmacologic
strategy has been utilized successfully to improve
patient outcome. The paucity of effective drug therapy
and poor prognosis in these patients indicates the
mechanisms underlying inception, propagation and
30 resolution of the disease are not well understood.

Since the formation of inflammatory gaps in
pulmonary endothelial cells is characteristic of
inflammation, any methods that can decrease these

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inflammatory gaps could be useful in treating and/or
preventing inflammation.


## SUMMARY OF THE INVENTION

5       The subject invention provides such a method which
involves the transient receptor potential (trp) gene.
More particularly, the invention provides a method of
decreasing inflammatory gaps in pulmonary endothelial
cells, the method comprising decreasing levels of
10   functional transient receptor potential gene product
(TRP) in the cells.  The invention further provides a
method of treating or preventing an inflammatory
condition in a subject, the method comprising
administering to the subject an amount of a compound
15   effective to decrease levels of functional transient
receptor potential gene product in the cells of the
subject.


## BRIEF DESCRIPTION OF THE DRAWINGS

20       These and other features and advantages of this
invention will be evident from the following detailed
description of preferred embodiments when read in
conjunction with the accompanying drawings in which:
        Figs. 1A-1C illustrate store-operated $Ca^{2+}$ entry in
25   rat (R) pulmonary arterial endothelial cells (PAECs). 1A:
individual traces from cells challenged with thapsigargin
(TG; $1\mu M$) in presence of 2 mM extracellular $Ca^{2+}$
concentration ($[Ca^{2+}]_o$).  1B: representative trances
comparing TG-induced cytosolic $Ca^{2+}$ concentration ($[Ca^{2+}]_i$)
30   response in presence (solid line0 and absence (dashed
line) of $[Ca^{2+}]_o$.  $[Ca^{2+}]_o$ was increased from 100 nM to 2 mM
at time indicated by $Ca^{2+}$.  Arrows, time of addition.  1C:
averaged data from all experiments conducted in presence
($\eta$ = 5 cells; open bars) and absence ($\eta$ = 5 cells; solid

bars) of $[Ca^{2+}]_o$ $t$, Time. *Significant difference compared with respective baseline fluorescence ratio of $Ca^{2+}$-bound (340-nm) to $Ca^{2+}$-unbound (380-nm) excitation wavelengths emitted at 510 nm (340/380), $P < 0.05$;

5      Figs. 2A-2B illustrate currents in RPAECs which were measured under whole cell configuration with bath and pipette solutions given in METHODS. Average ($\pm$SE) currents were calculated from last 20 ms of each 200-ms voltage step. 2A: summary of current voltage recordings

10  for unstimulated ($\eta$ = 4) and thapsigargin-treated (1 $\mu$M thapsigargin in pipette; $\eta$ = 8) RPAECs normalized to membrane capacitance to yield current density. *Insets*: sample sets of current pulses. 2B: net inward current density generated in response to thapsigargin;

15      Figs. 3A-3D illustrate scanning electron micrographs of RPAEC monolayers (X2,000). 3A: control (2 mM $[Ca^{2+}]_o$). 3B: thapsigargin treatment. 3C: RPAECs in 100 nM $[Ca^{2+}]_o$ challenged with thapsigargin. 3D: readdition of 2 mM $[Ca^{2+}]_o$ to RPAECs treated as in C;

20      Figs. 4A-4B illustrate the effect of $[Ca^{2+}]_o$ on F-actin distribution. Confocal microscopy was performed on 0.3-$\mu$m sections. Three micrographs/treatment are shown. 4A: unchallenged RPAEC monolayers in 2 mM $[Ca^{2+}]_o$. 4B: RPAECs incubated in 100 nM $[Ca^{2+}]_o$. 1: cross section

25  through tops of cells. In 2 mM $[Ca^{2+}]_o$ staining appeared as a peripheral band with apparent cell-to-cell contact sites. In low $[Ca^{2+}]_o$, diffuse punctate staining was observed throughout cells, but contact sites between cells were still obvious. II and III: cross sections

30  through middle and lower aspects of cells, respectively. In 2 mM $[Ca^{2+}]_o$, F-actin aligned in radiating strands, with obvious F-actin-containing focal contact sites. Low $[Ca^{2+}]_o$ was characterized by diffuse staining throughout, with cell junction integrity still intact;

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Figs. 5A-5C illustrate the ffect of thapsigargin on
F-actin organization in presence of 2 mM (5A), 100 nM
(5B), and 100 nM $[Ca^{2+}]_o$ followed by restoration of $[Ca^{2+}]_o$
to 2 mM (5C).  Confocal microscopy was performed on 0.3-
5  $\mu$m sections.  Three micrographs/ treatment are shown.  I:
cross section through upper portion of cells.  In
presence of 2 mM $[Ca^{2+}]_o$, diffuse perinuclear staining is
evident.  In low $[Ca^{2+}]_o$, a peripheral actin band with
cell-to-cell contact sites is prominent.  This peripheral
10  band retracted after $[Ca^{2+}]_o$ was readded, and
intercellular actin projections are discernible.  II and
III: cross sections of middle and lower portions of
cells, respectively.  In presence of 2 mM $[Ca^{2+}]_o$,
peripheral (cortical) actin band is absent, and F-actin
15  appears to align in stress fibers.  In low $[Ca^{2+}]_o$,
diffuse punctate staining is observed, but cortical actin
band is still present.  On readdition of $[Ca^{2+}]_o$, stress
fiber formation is obvious.

20              **DETAILED DESCRIPTION OF THE INVENTION**
The subject invention is based on the discovery that
decreasing levels of functional transient receptor
potential gene product (TRP) in a cell (such as by
decreasing transient receptor potential (trp) gene
25  expression or by decreasing activity of TRP or by
decreasing formation of calcium channels by TRP) can
decrease inflammatory gaps in pulmonary endothelial
cells.  Inflammatory gaps occur between pulmonary
endothelial cells due to an increase in intracellular
30  calcium in the cells.  The increase in intracellular
calcium in the cells occurs via TRP calcium channels.
Decreasing levels of functional TRP can therefore be used
to decrease intracellular calcium and therefore decrease
gap formation and therefore decrease inflammation.

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TRPs form calcium channels which belong to the
family of store-activated calcium channels.  One or more
isoforms of TRP may be required to form a functional
calcium channel.  Decreasing "levels" of functional TRP
5  refers to decreasing expression of the trp gene,
decreasing activity of the TRP such as by inhibiting one
or more TRP isoforms, and/or decreasing the formation of
active membrane-spanning calcium channels by the TRP.

The invention thus provides a method of decreasing
10  inflammatory gaps in pulmonary endothelial cells, the
method comprising decreasing levels of TRP in the cells.

Levels of TRP in the cells can be decreased by
various methods, at the gene and protein and "functional
calcium channel" levels.  In one embodiment, the levels
15  are decreased by decreasing trp gene expression of the
TRP in the cells.  This can be accomplished by exposing
the cells to a compound which decreases trp gene
expression of the TRP.  The compound could be, for
example, an antisense oligonucleotide targeted to the trp
20  gene.

In a similar embodiment, the compound which
decreases trp gene expression of the TRP could be a  .
ribozyme, which is a special category of antisense RNA
molecule having a recognition sequence complementary to
25  the mRNA encoding the TRP.  A ribozyme not only complexes
with a target sequence via complementary antisense
sequences, but also catalyzes the hydrolysis, or
cleavage, of the template mRNA molecule.  The expression
of the TRP protein is therefore prevented.

30      Other methods for decreasing trp gene expression
could also involve site-directed mutagenesis of the trp
gene to prevent expression of the TRP, or various gene
therapy techniques.

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Levels, in particular activity, of TRP in the cell can also be decreased by exposing the cells to an inhibitor of the TRP. Currently known inhibitors of voltage gated calcium channels include, for example,

5 nifedipine, nitrendipine, verapamil, and related compounds. Other inhibitors of the TRP could also readily be identified by various screening methods used in the art (see more detailed discussion below). In addition to chemical inhibitors, peptide inhibitors could

10 also be identified with currently known screening methods (for example, using phage display libraries and other peptide screening methods).

Levels of TRP in the cell can also be decreased by exposing the cells to a compound which interferes with

15 membrane calcium channel formation by the TRP.

Since the method of the subject invention is a method of decreasing inflammatory gaps in pulmonary endothelial cells, the cells of interest can be of human or animal origin.

20 The invention further provides a method of treating or preventing an inflammatory condition in a subject, the method comprising administering to the subject an amount of a compound effective to decrease levels of TRP in the cells of the subject. As above, the compound may

25 decrease levels of TRP by decreasing trp gene expression of the TRP, or by inhibiting the TRP, or by interfering with membrane calcium channel formation by the TRP.

The method is useful in an inflammatory condition. Examples of inflammatory conditions include regional

30 inflammatory disorders, such as asthma (late phase), pancreatitis, inflammatory bowel disease (IBD), peritonitis, rheumatoid arthritis, osteoarthritis, myocardial infarction, ocular inflammatory states, and stroke. Examples of inflammatory conditions also include

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systemic inflammatory disorders, such as systemic
inflammatory response syndrome (SIRS), cardiogenic shock,
adult respiratory distress syndrome (ARDS), multiple-
organ dysfunction (MOD), septic shock, and infant
5  respiratory distress syndrome (IRDS).
     In one embodiment, the invention employs
oligonucleotides targeted to nucleic acids encoding
functional transient receptor potential gene product
(TRP). The relationship between an oligonucleotide and
10  its complementary nucleic acid target to which it
hybridizes is commonly referred to as "antisense".
"Targeting" an oligonucleotide to a chosen nucleic acid
target, in the context of this invention, is a multistep
process. The process usually begins with identifying a
15  nucleic acid sequence whose function is to be modulated.
In the subject invention, this may be, for example, the
cellular gene (or mRNA made from the gene) for trp; i.e.,
the target is a nucleic acid encoding TRP, the trp gene,
or mRNA expressed from the trp gene. The targeting
20  process also includes determination of a site or sites
within the nucleic acid sequence for the oligonucleotide
interaction to occur such that the desired effect,
modulation of gene expression, will result. Once the
target site or sites have been identified,
25  oligonucleotides are chosen which are sufficiently
complementary to the target, i.e., hybridize sufficiently
well and with sufficient specificity, to give the desired
modulation.
     In the context of this invention "modulation" means
30  either inhibition or stimulation. Inhibition of trp gene
expression is presently the preferred form of modulation.
This modulation can be measured in ways which are routine
in the art, for example by Northern blot assay of mRNA
expression or Western blot assay of protein expression.

Effects on inflammatory gaps between cells can also be
measured, as taught in the examples of the instant
application. "Hybridization", in the context of this
invention, means hydrogen bonding, also known as Watson-
5 Crick base pairing, between complementary bases, usually
on opposite nucleic acid strands or two regions of a
nucleic acid strand. Guanine and cytosine are examples
of complementary bases which are known to form three
hydrogen bonds between them. Adenine and thymine are
10 examples of complementary bases which form two hydrogen
bonds between them. "Specifically hybridizable" and
"complementary" are terms which are used to indicate a
sufficient degree of complementarity such that stable and
specific binding occurs between the DNA or RNA target and
15 the oligonucleotide. It is understood that an
oligonucleotide need not be 100% complementary to its
target nucleic acid sequence to be specifically
hybridizable. An oligonucleotide is specifically
hybridizable when binding of the oligonucleotide to the
20 target interferes with the normal function of the target
molecule to cause a loss of utility, and there is a
sufficient degree of complementarity to avoid non-
specific binding of the oligonucleotide to non-target
sequences under conditions in which specific binding is
25 desired, i.e., under physiological conditions in the case
of in vivo assays or therapeutic treatment or, in the
case of in vitro assays, under conditions in which the
assays are conducted.

In various embodiments of this invention,
30 oligonucleotides are provided which are targeted to mRNA
encoding TRP. In accordance with this invention, persons
of ordinary skill in the art will understand that mRNA
includes not only the coding region which carries the
information to encode a gene product using the three

letter genetic code, including the translation start and
stop codons, but also associated ribonucleotides which
form a region known to such persons as the 5'-
untranslated region, the 3'-untranslated region, the 5'
5   cap region, intron regions and intron/exon or splice
junction ribonucleotides.  Thus, oligonucleotides may be
formulated in accordance with this invention which are
targeted wholly or in part to these associated
ribonucleotides as well as to the coding ribonucleotides.
10  The functions of mRNA to be interfered with include all
vital functions such as translocation of the RNA to the
site for protein translation, actual translation of
protein from the RNA, splicing or maturation of the RNA
and possibly even independent catalytic activity which
15  may be engaged in by the RNA.  The overall effect of such
interference with the RNA function is to cause
interference with trp gene expression.

In the context of this invention, the term
"oligonucleotide" refers to an oligomer or polymer of
20  nucleotide or nucleoside monomers consisting of naturally
occurring bases, sugars and intersugar (backbone)
linkages.  The term "oligonucleotide" also includes
oligomers comprising non-naturally occurring monomers, or
portions thereof, which function similarly.  Such
25  modified or substituted oligonucleotides are often
preferred over native forms because of properties such
as, for example, enhanced cellular uptake and increased
stability in the presence of nucleases.

The compounds and/or inhibitors used in the methods
30  of the subject invention encompass any pharmaceutically
acceptable salts, esters, or salts of such esters, or any
other compound/inhibitor which, upon administration to an
animal including a human, is capable of providing
(directly or indirectly) the biologically active

metabolite or residue thereof. Accordingly, for example, the disclosure is also drawn to prodrugs and pharmaceutically acceptable salts of the compounds and/or inhibitors used in the subject invention,

5  pharmaceutically acceptable salts of such prodrugs, and other bioequivalents.

In regard to prodrugs, the compounds and/or inhibitors for use in the invention may additionally or alternatively be prepared to be delivered in a prodrug

10  form. The term prodrug indicates a therapeutic agent that is prepared in an inactive form that is converted to an active form (i.e., drug) within the body or cells thereof by the action of endogenous enzymes or other chemicals and/or conditions.

15  In regard to pharmaceutically acceptable salts, the term pharmaceutically acceptable salts refers to physiologically and pharmaceutically acceptable salts of the compounds and/or inhibitors used in the subject invention: i.e., salts that retain the desired biological

20  activity of the parent compound and do not impart undesired toxicological effects thereto.

The oligonucleotides used in the method of the subject invention preferably are from about 8 to about 50 nucleotides in length. In the context of this invention

25  it is understood that this encompasses non-naturally occurring oligomers, preferably having 8 to 50 monomers.

The oligonucleotides used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis.

30  Equipment for such synthesis is sold by several vendors including Applied Biosystems. Any other means for such synthesis may also be employed; the actual synthesis of the oligonucleotides is well within the skill of the art. It is also well known to use similar techniques to

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prepare other oligonucleotides such as the
phosphorothioates and alkylated derivatives.  It is also
well known to use similar techniques and commercially
available modified amidites and controlled-pore glass
5    (CPG) products such as biotin, fluorescein, acridine or
psoralen-modified amidites and/or CPG (available from
Glen Research, Sterling VA) to synthesize fluorescently
labeled, biotinylated or other modified oligonucleotides
such as cholesterol-modified oligonucleotides.
10        In the context of this invention, to "expose" cells
(including the cells of tissues) to a compound and/or
inhibitor means to add the compound and/or inhibitor,
usually in a liquid carrier, to a cell suspension or
tissue sample, either in vitro or ex vivo, or to
15   administer the compounds and/or inhibitor to cells or
tissues within an animal (including a human) subject.
         For therapeutics, methods of decreasing inflammatory
gaps in pulmonary endothelial cells and methods of
preventing and treating inflammatory conditions are
20   provided.  The formulation of therapeutic compositions
and their subsequent administration is believed to be
within the skill in the art.  In general, for
therapeutics, a patient suspected of needing such therapy
is given a compound and/or inhibitor in accordance with
25   the invention, commonly in a pharmaceutically acceptable
carrier, in amounts and for periods which will vary
depending upon the nature of the particular disease, its
severity and the patient's overall condition.  The
pharmaceutical compositions may be administered in a
30   number of ways depending upon whether local or systemic
treatment is desired and upon the area to be treated.
Administration may be topical (including ophthalmic,
vaginal, rectal, intranasal, transdermal), oral or
parenteral.  Parenteral administration includes

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intravenous drip or infusion, subcutaneous, intraperitoneal or intramuscular injection, pulmonary administration, e.g., by inhalation or insufflation (especially relevant for treatment of asthma), or
5  intrathecal or intraventricular administration.

Formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or
10 oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful.

Compositions for oral administration include powders or granules, suspensions or solutions in water or non-
15 aqueous media, capsules, sachets or tablets. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable.

Compositions for parenteral, intrathecal or intraventricular administration may include sterile
20 aqueous solutions which may also contain buffers, diluents and other suitable additives.

In addition to such pharmaceutical carriers, cationic lipids may be included in the formulation to facilitate oligonucleotide uptake. One such composition
25 shown to facilitate uptake is Lipofectin (BRL, Bethesda MD).

Dosing is dependent on severity and responsiveness of the condition to be treated, with course of treatment lasting from several days to several months or until a
30 cure is effected or a diminution of disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates.

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Optimum dosages may vary depending on the relative
potency of individual compounds and/or inhibitors, and
can generally be calculated based on $IC_{50}$'s or $EC_{50}$'s in in
vitro and in vivo animal studies. For example, given the
5 molecular weight of compound (derived from
oligonucleotide sequence and/or chemical structure) and
an effective dose such as an $IC_{50}$, for example (derived
experimentally), a dose in mg/kg is routinely calculated.

The nucleic acid and amino acid sequences of various
10 transient receptor potential genes are known and readily
available from GenBank and described in the literature.
For example, see GenBank Accession No. NP 003295 which
discloses the 759 amino acid sequence of the human
transient receptor potential channel 1, GenBank Accession
15 No. AAC16725 which discloses the 123 amino acid sequence
of the rat transient receptor potential protein 1,
GenBank Accession No. AAD22978 which discloses the 778
amino acid sequence of the African clawed frog cation
channel TRP-1, GenBank Accession Nos. NM 003304 and
20 X89066 which each disclose the 4085 bp sequence of the
mRNA for human TRPC1 protein, GenBank Accession No.
AF061873 which discloses the 369 bp partial sequence of
the mRNA for rat trp1, GenBank Accession No. U40980 which
discloses the 372 bp partial sequence of the mRNA for
25 house mouse trp-related protein 1, and GenBank Accession
No. X90696 which discloses the 304 bp sequence of the
mRNA for the African clawed frog trp-like protein. See
also, Wes et al., Proc Natl Acad Sci USA 92(21):9652-9656
(Oct 10, 1995).
30      Given these sequences, one can design appropriate
antisense molecules for use in the subject invention.
Furthermore, by expressing the functional TRP calcium
channel in a host cell, one can screen for suitable
compounds and/or inhibitors for use in the subject

invention.  The function of the encoded calcium channel
can be assayed according to methods known in the art,
such as by analysis of the channel following the
functional expression of the channel in oocytes of the
5  frog *Xenopus laevis*.  As used herein, "functional"
expression refers to the synthesis and any necessary
post-translational processing of a calcium channel
molecule in a cell so that the channel is inserted
properly in the cell membrane and is capable of
10  conducting calcium ions in accordance with a store-
activated channel.

More particularly, having known nucleic acid
molecules encoding the TRP, a method for screening a
chemical agent (compound or inhibitor) for the ability of
15  the chemical agent to modify calcium channel function
begins by introducing the nucleic acid molecule encoding
the TRP into a host cell, and expressing the TRP encoded
by the molecule in the host cell.  The expression results
in the functional expression of a TRP calcium channel in
20  the membrane of the host cell.  The cell is then exposed
to a chemical agent and evaluated to determine if the
chemical agent modifies the function of the TRP calcium
channel.  From this evaluation, chemical agents effective
in altering the function of the sodium channel can be
25  found and utilized in the methods of the subject
invention.

Drugs, such as peptide drugs, which inhibit the TRP
or which interfere with function TRP calcium channel
formation can be made using various methods known in the
30  art.  Initially, a monoclonal antibody can be prepared
which specifically hybridizes to the TRP, thereby
interfering with activity and/or channel formation.

The monoclonal antibodies can be produced by
hybridomas.  A hybridoma is an immortalized cell line

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which is capable of secreting a specific monoclonal
antibody.

In general, techniques for preparing polyclonal and
monoclonal antibodies as well as hybridomas capable of
5   producing the desired antibody are well known in the art
(see Campbell, A.M., "Monoclonal Antibody Technology:
Laboratory Techniques in Biochemistry and Molecular
Biology", Elsevier Science Publishers, Amsterdam, The
Netherlands (1984); St. Groth, et al., J Immunol Methods
10  35:1-21 (1980)).  Any animal (mouse, rabbit, etc.) which
is known to produce antibodies can be immunized with the
TRP (or an antigenic fragment thereof).  Methods for
immunization are well known in the art.  Such methods
include subcutaneous or intraperitoneal injection of the
15  TRP.  One skilled in the art will recognize that the
amount of the TRP used for immunization will vary based
on the animal which is immunized, the antigenicity of the
TRP, and the site of injection.

The TRP which is used as an immunogen may be
20  modified or administered in an adjuvant in order to
increase the TRP's antigenicity.  Methods of increasing
the antigenicity of a protein are well known in the art
and include, but are not limited to, coupling the antigen
with a heterologous protein (such as a globulin or beta-
25  galactosidase) or through the inclusion of an adjuvant
during immunization.

For monoclonal antibodies, spleen cells from the
immunized animals are removed, fused with myeloma cells,
such as SP2/O-Ag 15 myeloma cells, and allowed to become
30  monoclonal antibody producing hybridoma cells.

Any one of a number of methods well known in the art
can be used to identify the hybridoma cell which produces
an antibody with the desired characteristics.  These
include screening the hybridomas with an ELISA assay,

western blot analysis, or radioimmunoassay (Lutz, et al.,
Exp Cell Res 175:109-124 (1988)).

Hybridomas secreting the desired antibodies are
cloned and the class and subclass are determined using
5  procedures known in the art (Campbell, A.M., "Monoclonal
Antibody Technology: Laboratory Techniques in
Biochemistry and Molecular Biology", Elsevier Science
Publishers, Amsterdam, The Netherlands (1984)).

For polyclonal antibodies, antibody containing
10  antisera is isolated from the immunized animal and is
screened for the presence of antibodies with the desired
specificity using one of the above-described procedures.

Once a monoclonal antibody which specifically
hydridizes to the TRP is identified, the monoclonal
15  (which is itself a compound or inhibitor which can be
used in the subject invention) can be used to identify
peptides capable of mimicking the inhibitory activity of
the monoclonal antibody.  One such method utilizes the
development of epitope libraries and biopanning of
20  bacteriophage libraries.  Briefly, attempts to define the
binding sites for various monoclonal antibodies have led
to the development of epitope libraries.  Parmley and
Smith developed a bacteriophage expression vector that
could display foreign epitopes on its surface (Parmley,
25  S.F. & Smith, G.P., Gene 73:305-318 (1988)).  This vector
could be used to construct large collections of
bacteriophage which could include virtually all possible
sequences of a short (e.g. six-amino-acid) peptide.  They
also developed biopanning, which is a method for
30  affinity-purifying phage displaying foreign epitopes
using a specific antibody (see Parmley, S.F. & Smith,
G.P., Gene 73:305-318 (1988); Cwirla, S.E., et al., Proc
Natl Acad Sci USA 87:6378-6382 (1990); Scott, J.K. &
Smith, G.P., Science 249:386-390 (1990); Christian, R.B.,

et al., J Mol Biol 227:711-718 (1992); Smith, G.P. &
Scott, J.K., Methods in Enzymology 217:228-257 (1993)).

After the development of epitope libraries, Smith et
al. then suggested that it should be possible to use the

5  bacteriophage expression vector and biopanning technique
of Parmley and Smith to identify epitopes from all
possible sequences of a given length. This led to the
idea of identifying peptide ligands for antibodies by
biopanning epitope libraries, which could then be used in

10 vaccine design, epitope mapping, the identification of
genes, and many other applications (Parmley, S.F. &
Smith, G.P., Gene 73:305-318 (1988); Scott, J.K., Trends
in Biochem Sci 17:241-245 (1992)).

Using epitope libraries and biopanning, researchers

15 searching for epitope sequences found instead peptide
sequences which mimicked the epitope, i.e., sequences
which did not identify a continuous linear native
sequence or necessarily occur at all within a natural
protein sequence. These mimicking peptides are called

20 mimotopes. In this manner, mimotopes of various binding
sites/proteins have been found.

The sequences of these mimotopes, by definition, do
not identify a continuous linear native sequence or
necessarily occur in any way in a naturally-occurring

25 molecule, i.e. a naturally occurring protein. The
sequences of the mimotopes merely form a peptide which
functionally mimics a binding site on a naturally-
occurring protein.

Many of these mimotopes are short peptides. The

30 availability of short peptides which can be readily
synthesized in large amounts and which can mimic
naturally-occurring sequences (i.e. binding sites) offers
great potential application.

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Using this technique, mimotopes to a monoclonal antibody that recognizes TRP can be identified. The sequences of these mimotopes represent short peptides which can then be used in various ways, for example as

5  peptide drugs that bind to TRP and decrease the activity of TRP. Once the sequence of the mimotope is determined, the peptide drugs can be chemically synthesized.

The peptides for use in the subject invention can contain any naturally-occurring or

10  non-naturally-occurring amino acids, including the D-form of the amino acids, amino acid derivatives and amino acid mimics, so long as the desired function and activity of the peptide is maintained. The choice of including an (L)- or a (D)-amino acid in the peptide depends, in part,

15  on the desired characteristics of the peptide. For example, the incorporation of one or more (D)-amino acids can confer increased stability on a peptide and can allow a peptide to remain active in the body for an extended period of time. The incorporation of one or more

20  (D)-amino acids can also increase or decrease the pharmacological activity of a peptide.

The peptide may also be cyclized, since cyclization may provide the peptide with superior properties over their linear counterparts.

25  Modifications to the peptide backbone and peptide bonds thereof are encompassed within the scope of amino acid mimic or mimetic. Such modifications can be made to the amino acid, derivative thereof, non-amino acid moiety or the peptide either before or after the amino acid,

30  derivative thereof or non-amino acid moiety is incorporated into the peptide. What is critical is that such modifications mimic the peptide backbone and bonds which make up the same and have substantially the same spacial arrangement and distance as is typical for

traditional peptide bonds and backbones.  An example of
one such modification is the reduction of the carbonyl(s)
of the amide peptide backbone to an amine.  A number of
reagents are available and well known for the reduction
5    of amides to amines such as those disclosed in Wann et
al., JOC 46:257 (1981) and Raucher et al., Tetrahedron
Lett 21:14061 (1980).  An amino acid mimic is, therefore,
an organic molecule that retains the similar amino acid
pharmacophore groups as are present in the corresponding
10   amino acid and which exhibits substantially the same
spatial arrangement between functional groups.

The substitution of amino acids by non-naturally
occurring amino acids and amino acid mimics as described
above can enhance the overall activity or properties of
15   an individual peptide thereof based on the modifications
to the backbone or side chain functionalities.  For
example, these types of alterations can enhance the
peptide's stability to enzymatic breakdown and increase
biological activity.  Modifications to the peptide
20   backbone similarly can add stability and enhance
activity.

One skilled in the art, using the identified
sequences can easily synthesize the peptides for use in
the invention.  Standard procedures for preparing
25   synthetic peptides are well known in the art.  The novel
peptides can be synthesized using: the solid phase
peptide synthesis (SPPS) method of Merrifield, J Am Chem
Soc 85:2149 (1964) or modifications of SPPS; or, the
peptides can be synthesized using standard solution
30   methods well known in the art (see, for example,
Bodanzsky, "Principles of Peptide Synthesis", 2d Ed.,
Springer-Verlag (1993)). Alternatively, simultaneous
multiple peptide synthesis (SMPS) techniques well known
in the art can be used. Peptides prepared by the method

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of Merrifield can be synthesized using an automated
peptide synthesizer such as the Applied Biosystems
431A-01 Peptide Synthesizer (Mountain View, Calif.) or
using the manual peptide synthesis technique described by
5   Houghten, Proc Natl Acad Sci USA 82:5131 (1985).


METHODS
     *Isolation of RPAECs.* Male Sprague-Dawley rats (CD
strain, 350-400 g; Charles River) were euthanized by an
10  intraperitoneal injection of 50 mg of pentobarbital
sodium (Nembutal, Abbott Laboratories, Chicago, IL).
After sternotomy, the heart and lungs were removed en
bloc, and the pulmonary arterial segment between the
heart and lung hili was dissected, split, and fixed onto
15  a 35-mm plastic dish.  Endothelial cells were obtained by
gentle intimal scraping with a plastic cell lifter and
were seeded into a 100-mm petri dish containing 10 ml of
seeding medium (~1:1 DMEM-Ham's F-12 + 10% fetal bovine
serum) (37).  Afer incubation for 1 wk (21% $O_2$-5% $CO_2$-74%
20  $N_2$ at 37°C), smooth muscle cell contaminants were marked
and then removed by pipette aspiration.  Cells were
verified as endothelial by positive factor VIII staining
and uptake of 1,1'-dioctadecyl-3,3,3',3'-
tetramethylindocarbocyanine perchlorate (DiI)-labeled
25  acetylated low-density lipoprotein.  When the primary
culture reached confluence, cells were passaged by
trypsin digestion into T-75 culture flasks (Corning), and
standard tissue culture techniques were followed until
the cells were ready for experimentation (passages 6-20).
30       *$[Ca^{2+}]_i$ measurement by fura 2 fluorescence.*  RPAECs
were seeded onto chambered glass coverslips (Nalge Nunc,
Naperville, IL) and grown to confluence. $[Ca^{2+}]_i$ was
estimated with the $Ca^{2+}$-sensitive fluorophore fura 2-AM
(Molecular Probes, Eugene, OR) according to methods

previously described by our laboratory (38). Because
this is the first report of $[Ca^{2+}]_i$ measurements in
cultured RPAECs, a summary of the technique will be
presented. RPAECs were washed with 2 ml of a HEPES
5  (Fisher Scientific, Atlanta, GA)-buffered physiological
salt solution (PSS) containing (in g/l) 6.9 NaCl, 0.35
KCl, 0.16 $KH_2PO_4$, 0.141 $MgSO_4$, 2.0 D-glucose, and 2.1
$NaHCO_3$. The loading solution (1 ml) consisted of PSS plus
3 $\mu M$ fura 2-AM, 3 $\mu l$ of a 10% pluronic acid solution, and
10  2 mM or 100 nM $CaCl_2$. RPAECs were fura loaded for 20 min
in a $CO_2$ incubator at 37°C. After this loading period,
the cells were again washed with PSS (2 ml) and treated
with deesterification medium (PSS + 2 mM or 100 nM $CaCl_2$)
for an additional 20 min. After deesterification, $[Ca^{2+}]_i$
15  was assessed with an Olympus 1X70 inverted microscope at
x400 with a xenon arc lamp photomultiplier system (Photon
Technologies, Monmouth Junction, NJ), and data were
acquired and analyzed with PTI Felix software.
Epifluorescence (signal averaged) was measured from three
20  to four endothelial cells in a confluent monolayer, and
the changes in $[Ca^{2+}]_i$ are expressed as the fluorescence
ratio of the $Ca^{2+}$-bound (340-nm) to $Ca^{2+}$-unbound (380-nm)
excitation wavelengths emitted at 510 nm.

   *Electrophysiological determination of store-operated*
25  $Ca^{2+}$ *entry.* Whole cell patch clamp was utilized to
measure transmembrane ion flux in thapsigargin-stimulated
RPAECs. Confluent RPAECs were enzyme dispersed, seeded
onto 35-mm plastic culture dishes, and then allowed to
reattach for at least 24 h before patch-clamp experiments
30  were performed. Single RPAECs exhibiting a flat,
polyhedral morphology were studied. These cells were
chosen for study because their morphology was consistent
with RPAECs from a confluent monolayer. The
extracellular and pipette solutions were composed of the

following (in mM): 1) extracellular: 110
tetraethylammonium aspartate, 10 calcium aspartate, 10
HEPES, and 0.5 3,4-diaminopyridine; and 2) pipette: 130
$N$-mehyl-D-glucamine, 1.15 EGTA, 10 HEPES, 1 Ca(OH)$_2$, and 2

5   Mg$^{2+}$-ATP. Both solutions were adjusted to 290-300 mosM
with sucrose and pH 7.4 with methane sulfonic acid, and
[Ca$^{2+}$]$_i$ was estimated as 100 nM (10). The pipette
resistance was 2-5 MΩ. Data were obtained with a HEKA
EPC9 amplifier (Lambrecht/Pfaltz) and sampled on-line

10  with Pulse + Pulsefit software (HEKA). All recordings
were made at room temperature (22°C). To generate
current-voltage (I-V) relationships, voltage pulses were
applied from -100 to +100 mV in 20-mV increments, with a
200-ms duration during each voltage step and a 2-s

15  interval between steps. The holding potential between
each step was 0 mV.

    *Assessment of endothelial cell shape change.* RPAECs
were seeded onto 35-mm plastic culture dishes and grown
to confluence. Growth medium was replaced with

20  experimental buffer (same as that used for [Ca$^{2+}$]$_i$
measurements but without fura 2), and the cells were
subjected to one of the following protocols: 1) vehicle
control (5 min) in 2 mM or 100 nM extracellular Ca$^{2+}$
concentration ([Ca$^{2+}$]$_o$); 2) thapsigargin (1 μM, 5 min) in

25  2 mM or 100 nM ([Ca$^{2+}$]$_o$; or 3) thapsigargin in 100 nM
[Ca$^{2+}$]$_o$ + readdition of 2 mM CaCl$_2$ (5 min). At the end of
each experiment, the cell monolayers were fixed in 3%
gluteraldehyde-PBS for 2 h. The cells were washed two
times with 0.1 M cacodylate buffer, dehydrated by

30  immersion in a series of ethanol dilutions, critical
point dried in CO$_2$, and covered with 20 nm of gold.
Specimens were viewed at 10 kW at a 15° inclination.
Scanning electron micrographs were taken of

representative areas in the monolayer by a pathologist
blinded to the experimental protocols.

    *Assessment of filamentous actin arrangement.*
Experiments to determine filamentous actin (F-actin)
5  arrangement were conducted in parallel with those
assessing endothelial cell shape. RPAECs were seeded
onto glass coverslips, and F-actin was stained with
Oregon Green-phalloidin (Molecular Probes) with a
standard fixation and staining protocol. Cells were
10  analyzed by confocal microscopy (excitation at 496 nm and
emmision at 520 nm). Micrographs were taken at multiple
cellular depths (0.3-$\mu$m steps, 13-15 sections) and were
used to deduce the microfilamentous cytoskeleton
configurations of the cells.

15    *Identification of trp gene products in pulmonary
endothelial cells.* For RT-PCR cloning experiments,
RPAECs and human (H) PAECs (Clonetics, San Diego, CA)
were studied. Standard techniques for RT-PCR subcloning
were followed. All chemical reagents used were of
20  molecular biological grade. Briefly, total RNA was
extracted from RPAECs and HPAECs grown to confluence in
75-cm$^2$ culture flasks (~10$^7$ cells) with RNA Stat-60 (Tel-
Test "B", Friendswood, TX). First-strand synthesis was
performed with reverse transcriptase and oligo(dT) primer
25  (GIBCO BRL) on 1 $\mu$g of DNase I-treated total RNA. PCR was
then performed with the following sets of primers: 1)
Trp1: 5'- TCG CCG AAC GAG GTG ATG G-3' (sense) and 5'-GTT
ATG GTA ACA GCA TTT CTC C-3' (antisense), 2) Trp3: 5'-ACC
TCT CAG GCC TAA GGG AG-3' (sense) and 5'-CCT TCT GAA GTC
30  TTC TCC TGC-3' (antisense), and 3) Trp6: 5'-CT ACA TTG
GCG CAA AAC AG-3' (sense) and 5'-CAC CAT ACA GAA CGT AGC
CG-3' (antisense). PCR products were ligated into pCR2.1
vectors (TA Cloning Kit, Invitrogen, San Diego, CA),
transformed into competent cells, and screened by PCR for

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proper inserts.   Bacterial cultures were grown for 16-18
h, and the plasmids were purified with Promega Wizard
Minipreps (Madison, WI).   Sequencing was performed by an
automated fluorescence sequencer (AB1370A), and deduced
5  amino acid alignments were carried out with the Blast
software program.


**EXAMPLE I**

Leakage of proteinaceous fluid from the vascular
10  spaces into intersitial spaces and, in severe forms, into
alveoli causes pulmonary edema in ARDS.   Such fluid
accumulation in the lung parenchyma de-oxygenates blood.
Hypoxemia combined with poor tissue perfusion severely
compromises organ function.
15     Disruption of the pulmonary endothelial cell barrier
is an initiating event that promotes edema.   Endothelial
disruption is a regulated process occurring secondary to
release of toxic oxygen radicals and proteases by white
blood cells, ischemia-reperfusion injury, and/or neuro-
20  humoral inflammatory and vasoactive mediators.   These
agents act as so-called first messengers to induce
endothelial cell contraction and decrease cell-cell and
cell-matrix tethering, resulting in gap formation between
cells that forms a paracellular pathway for transfer of
25  the proteinaceous fluid.   The underlying mechanisms
linking the host of first messengers to altered cell
shape are unknown.
    Cytosolic $Ca^{2+}$ ($[Ca^{2+}]i$) and adenosine 3',5' cyclic
monophosphate (cAMP) are two intracellular signals
30  importantly dictating endothelial cell-cell apposition,
and thus permissiveness of the endothelial barrier for
fluid transudation.   Increased cytosolic $Ca^{2+}$ ($[Ca^{2+}]i$)
engages the endothelial contractile apparatus to pull
cells inwardly.   In addition, increased $[Ca^{2+}]i$ uncouples

cell-cell and cell-matrix tethering so that inward
contractions produce focal gaps between cells in the
vessel wall.  Virtually all of these effects depend upon
$Ca^{2+}$ entry, but remarkably not a single gene product
5  encoding a $Ca^{2+}$ channel was originally identified in
endothelial cells.  Recently a Drosophila melanogaster
$Ca^{2+}$ channel called transient receptor potential, or Trp,
was shown to mediate $Ca^{2+}$ entry responsible for light
perception in retina.  Human homologues of this gene
10  product were then found to be expressed in endothelial
cells.

   While increased $[Ca^{2+}]i$ promotes disruption of the
endothelial cell barrier, increased cAMP enhances
endothelial cell barrier function.  Indeed, cAMP
15  elevating agents are commonly used in clinical medicine
for the treatment of inflammation.  Adenylyl cyclase (the
enzyme responsible for synthesis of cAMP) activators and
phosphodiesterase (the enzyme responsible for the
breakdown of cAMP) inhibitors have both been utilized to
20  increase cellular cAMP content for the treatment of
urticaria and asthma among other conditions.  Despite an
appreciation for the utility of these pharmacologic
strategies in treatment of various forms of inflammation,
the influence of inflammation on endothelial cell cAMP
25  content had not been carefully investigated.  Studies
indicated that during inflammation cAMP levels decrease
in endothelial cells, which permissively increases
permeability.  Interestingly, endothelial cells express a
form of adenylyl cyclase that is inhibited by $Ca^{2+}$ entry.
30  Thus, when inflammatory first messengers stimulate $Ca^{2+}$
entry into endothelial cells, cAMP content is reduced.

   Although Trp3 and Trp6 are not SOCs (6, 46), Trp1
may form SOCs based on the following experimental
evidence: 1) Trp1 and its splice variant TRPC1A increase

store-operated $Ca^{2+}$ entry when expressed in COS cells (45,
47) and 2) expression of antisense *trp* sequences in
murine L(tk⁻) cells greatly attenuates store-operated $Ca^{2+}$
entry evoked by Ins(1,4,5)$P_3$ (45). Information concerning
5  putative functions for Trp2, -4, and -5 are lacking in
the literature.

Because activation of store-operated $Ca^{2+}$ entry is
known to increase vascular permeability in isolated lungs
(9, 18), thereby suggesting that pulmonary endothelial
10 SOCs are important for regulation of endothelial barrier
integrity, studies were designed to characterize the
store-operated $Ca^{2+}$ entry pathway in rat (R) pulmonary
arterial endothelial cells (PAECs). The hypothesis was
that a functional consequence of activating endothelial
15 SOCs is a change in cell shape, leading to
interendothelial gap formation and cytoskeletal
rearrangement. To test this hypothesis, RPAECs were
challenged with thapsigargin, a plant alkaloid that
activates store-operated $Ca^{2+}$ entry independent of ligand-
20 receptor-G protein-coupled processes (40, 43), and the
changes in endothelial cell shape and microfilamentous
cytoskeletal arrangement were monitored. It was then
determined whether RPAECs express Trp1 in order to
address the possible molecular basis for the pulmonary
25 endothelial store-operated $Ca^{2+}$ entry pathway. Ther data
indicate that store-operated $Ca^{2+}$ entry promotes cell
shape change in rat pulmonary endothelial cells
expressing Trp1 and further suggest that $Ca^{2+}$ entry
through SOCs involves site-specific rearrangement of the
30 microfilamentous cytoskeleton.

*Thapsigargin activates store-operated* $Ca^{2+}$ *entry in
RPAECs.* Fura 2 epifluorescence was monitored, and as
shown in Fig. 1A and summarized in Fig. 1C (open bars),
RPAECs incubated in 2 mM $[Ca^{2+}]_o$ had baseline fluorescence

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ratios averaging 0.91 ± 0.02. Thapsigargin produced a gradual increase in $[Ca^{2+}]_i$ to a peak level followed by a modest decline, producing a new steady state, or plateau, in $[Ca^{2+}]_i$. Figure 1, B (dashed line) and C (solid bars),

5 illustrates that the thapsigargin-induced response was dependent on $[Ca^{2+}]_o$. When experiments were repeated in PSS containing 100 nM $[Ca^{2+}]_o$, the baseline fluorescence ratio value decreased slightly, and both the peak and sustained plateau phases of the thapsigargin-induced

10 response were significantly attenuated. Subsequence readdition of 2 mM $[Ca^{2+}]_o$ produced an immediate increase in $[Ca^{2+}]_i$, thereby illustrating functional store-operated $Ca^{2+}$ entry pathways.

The whole cell currents from RPAECs challenged with

15 thapsigargin were largely linear over a range of membrane potentials, although linearity was lost at about +40 mV. Figure 2A shows current densities recorded 3-5 min after the whole cell configuration was established. Under these experimental conditions (i.e., an $[Ca^{2+}]_o$-to-$[Ca^{2+}]_i$

20 ratio of $10^5$), RPAECs had a small, net inward $Ca^{2+}$ "leak" (control measurements without thapsigargin) calculated as 0.39 ± 0.43 pA/pF at -80 mV (by subtraction of the outward from the inward current at each membrane potential). Thapsigargin right shifted the $I$-$V$ curve and

25 increased the current magnitude (slope conductance = 1.64 nS, calculated from -100 to -20 mV without respect to cell capacitance). The net inward current stimulated by the thapsigargin was calculated as 5.45 ± 0.90 pA/pF at - 80 mV (Fig. 2B).

30 *Store-operated* $Ca^{2+}$ *entry evokes endothelial cell shape change and F-actin cytoskeletal rearrangement in RPAECs.* To determine a functional consequence of SOC activation in RPAECs, changes in endothelial cell shape and formation of intercellular gaps in thapsigargin-

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treated confluent RPAEC monolayers were assessed.
Because it was determined that SOC activation was
apparent 3-5 min after thapsigargin treatment,
endothelial morphology at this fixed time point was
5    studied.  Figure 3 shows scanning electron micrographs of
RPAECs after different treatments.  Untreated RPAECs
(Fig. 3A) in 2 mM $[Ca^{2+}]_o$ exhibited a characteristic
"cobblestone" morphology essentially devoid of
intercellular gaps.  Thapsigargin produced endothelial
10   cell retraction and intercellular gap formation (Fig.
3B).  The changes in endothelial cell morphology were
dependent on $[Ca^{2+}]_o$ because RPAECs incubated in 100 nM
$[Ca^{2+}]_o$ and challenged with thapsigargin displayed little
change in morphology and a lack of interendothelial gaps
15   (Fig. 3C).  The subsequent readdition of 2 mM $[Ca^{2+}]_o$ had
a dramatic effect on endothelial cell shape, causing
pronounced cell retraction and gap formation (Fig. 3D).
Thus, $Ca^{2+}$ entry through activated SOCs sufficiently
promoted endothelial cell shape alterations and
20   interendothelial gap formation.
     Because the actin cytoskeleton is pivotal for
determining endothelial cell shape, the arrangement of F-
actin in control and thapsigargin-treated RPAECs was
studied.  Figure 4A shows F-actin localization in
25   untreated RPAECs incubated with 2 mM $[Ca^{2+}]_o$.  Under these
conditions, cells contained dense peripheral actin bands
with apparent focal contact sites between cells.  Some
transcellular, centrally located filaments were also
seen.  Figure 4B shows that incubation of RPAECs in low
30   $[Ca^{2+}]_o$ alone had an effect on F-actin configuration.
Diffuse, punctate F-actin staining was observed centrally
in the cell, whereas densely stained focal sites at the
peripheral intercellular junctions were still obvious.
Thapsigargin-treated RPAECs incubated in 2 mM $[Ca^{2+}]_o$

(Fig. 5A) showed a decrease in peripheral F-actin density
and an increase in the number and/or density of central
transcellular F-actin filaments.  Actin-containing
projections could be seen spanning the interendothelial
gaps.  Thapsigargin administration to RPAECs incubated in
low $[Ca^{2+}]_o$ (Fig. 5B) produced only modest changes in F-
actin arrangements compared with incubation in low $[Ca^{2+}]_o$
alone.  However, the subsequent readdition of 2 mM $[Ca^{2+}]_o$
(Fig. 5C) produced the appearance of dense, transcellular
fibers and a decrease in peripheral F-actin staining.
Thus $[Ca^{2+}]_o$ appears to affect the localization of
intracellular F-actin, and $Ca^{2+}$ influx through activated
SOCs configures the microfilamentous cytoskeleton for the
alteration of cell shape.

   *RT-PCR reveals the presence of Trp1 in RPAECs.*
Three specific mammalian *trp* gene products, Trp1, Trp3,
and Trp6, were screened for because all are associated
with $Ca^{2+}$ influx into nonexcitable cell lines, although
only Trp1 appears to possess the functional capacity to
mediate store-operated $Ca^{2+}$ entry.  The Trp3 or Trp6
products from confluent RPAECs were not amplified.  To
determine whether this was a species-specific effect, RT-
PCR was performed with HPAECs but likewise detected
neither Trp3 nor Trp6 expression.  However, both products
could be amplified in rat brain, indicating that the
primers were capable of amplifying these *trp* gene
products (data not shown).  In contrast, RT-PCR products
for Trp1 were identified in both RPAECs and HPAECs.  The
RPAEC and HPAEC products were 96 and 100% homologous,
respectively, to the reported nucleotide sequence for
human Trp1 (Table A).  The deduced amino acid alignments
revealed 100% amino acid homology between both
endothelial products and human Trp1 over the region
studied (Table B).  Thus Trp1 is present and may

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contribute to RPAEC SOC formation, whereas Trp3 and Trp6
likely are not expressed in the pulmonary endothelium.

Although activation of $Ca^{2+}$ entry is sufficient to
induce the interendothelial cell gap formation necessary
5    for the transit of macromolecules and cells from blood
into tissue, the mode of $Ca^{2+}$ entry responsible for
changing cell shape is unknown.  Nonexcitable cells
possess store-operated $Ca^{2+}$ entry pathways.  Store-
operated $Ca^{2+}$ entry is activated in response to agonist-
10   induced stimulation of membrane phospholipases,
generation of $Ins(1,4,5)P_3$, $Ca^{2+}$ release from
intracellular stores, and subsequent lowering of store
$Ca^{2+}$ concentrations (4, 8, 13, 16, 31, 34, 35, 41, 42).
Presently, there are three prevailing questions regarding
15   store-operated $Ca^{2+}$ entry pathways.  1) What specific
cellular functions are regulated by $Ca^{2+}$ influx through
this pathway?  2) What is the molecular identity of the
membrane channels responsible for mediating store-
operated $Ca^{2+}$ entry?  3) What is the nature of the signal
20   linking $Ca^{2+}$ store depletion to store-operated $Ca^{2+}$ entry?
The present study addressed the first two of these three
important questions.

Thapsigargin was utilized to test store-operated $Ca^{2+}$
entry pathways because this agent produces intracellular
25   $Ca^{2+}$ store depletion without the confounding influences of
ligand-receptor-heterotrimeric G protein activation (40,
43, 47).  Fura 2-loaded RPAEC monolayers exhibited an
increased $[Ca^{2+}]_i$ that was dependent on $Ca^{2+}$ influx in
response to thapsigargin, thereby indicating the presence
30   of store-operated $Ca^{2+}$ entry pathways.  To begin
elucidating the electrophysiological characteristics of
RPAEC SOCs, whole cell patch clamp in single cells was
performed.  Intracellular and extracellular patch
solutions were performed to isolate thapsigargin-induced

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$Ca^{2+}$ currents and determine whether thapsigargin activated
a channel(s) responsible for $Ca^{2-}$ release-activated
current ($I_{CRAC}$) (16). However, the thapsigargin-induced
current measured under these experimental conditions was
5   not identical to $I_{CRAC}$ because significant outward current
was also measured.

It was possible that the total current measured in
response to thapsigargin reflected coactivation of both a
$Ca^{2+}$-selective cation channel and an anion channel because
10  aspartate was utilized to replace $Cl^-$ in the extracellular
solution, and aspartate has recently been shown to be
conducted through $Ca^{2+}$- and/or volume-activated $Cl^-$
channels (29). In support of this idea, *N*-
phenylanthranilic acid, a potent blocker of $Ca^{2+}$-activated
15  anion channels (27), had little effect on the inward
current observed at negative voltages but strongly
attenuated the outward current at positive voltages (data
not shown). Thus thapsigargin may activate an anion
channel capable of conducting large organic anions as
20  previously reported in bovine pulmonary endothelium (27,
29). When the anion conductance contribution to the
total thapsigargin-stimulated current is then considered,
a current analogous to $I_{CRAC}$ is apparent.

Activation of SOCs in RPAECs causes the appearance
25  of intercellular gaps and rounding of endothelial cells.
One intracellular target affected by SOC activation is
plasmalemmal-associated and centrally located F-actin.
It is accepted that changes in $[Ca^{2+}]_i$ lead to
reconfigurations of the microfilamentous cytoskeleton
30  (21, 22, 30), although it has previously been unclear
whether $Ca^{2+}$ release from intracellular stores or $Ca^{2+}$
influx is necessary to produce cytoskeletal changes
leading to cell shape change.

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Thapsigargin produced a loss of plasmalemmal F-actin
staining concurrent with an increase in central F-actin
staining. When store depletion alone was produced, i.e.,
thapsigargin in the absence of $[Ca^{2+}]_o$, rearrangement of
5   cortical actin fibers did not occur and less F-actin
staining was observed centrally. Under these conditions,
RPAECs did not respond to thapsigargin with a change in
cell shape. The readdition of $[Ca^{2+}]_o$ caused
morphological changes in both the peripheral (loss of
10  dense actin staining) and centrally located (increased
actin staining and transcellular filament formation) F-
actin pools, indicating that $Ca^{2-}$ influx through SOCs is
sufficient to adjust the microfilament system of the
cells to produce interendothelial gap formation. It is
15  presently unclear how $Ca^{2+}$ influx through SOCs
specifically regulates the endothelial F-actin
cytoskeleton, although a possible mediator of the $Ca^{2+}$
influx-induced cytoskeletal rearrangement is Rho, a
small-molecular-weight monomeric G protein, the activity
20  of which produces actin polymerization and stress fiber
formation (1, 14).

Interestingly, incubation of RPAEC monolayers in low
$[Ca^{2+}]_o$ alone caused rearrangement of central F-actin but
had no apparent effect on peripheral, or cortical, F-
25  actin. Under these conditions, $Ca^{2+}$ release could have
been promoted because a more favorable electrochemical
gradient for $Ca^{2+}$ to leak from intracellular stores
existed. Centrally located F-actin in close proximity to
$Ca^{2+}$ stores could have been affected by $Ca^{2+}$ release but
30  not in a manner sufficient to drive an active cell shape
change. Another possibility to consider with respect to
the F-actin rearrangement is that low $[Ca^{2+}]_o$ provided
less basal $Ca^{2+}$ influx that was somehow setting the F-
actin cytoskeletal architecture.

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The findings indicate that at least Trp1, but
neither Trp3 nor Trp6, is expressed in pulmonary
endothelial cells.  It is uncertain how *trp* gene products
may be organized in the membrane to form a functional
5  channel, but it has been proposed that SOCs may be
composed of *trp* homo- and/or heteromultimers (5).
Because the data indicate that neither Trp3 nor Trp6 are
present in rat or human pulmonary endothelial cells, the
SOC is not composed of Trp1-Trp3 or Trp1-Trp6
10  heteromultimers.

What are the implications of the observation that
SOC activation produces changes in PAEC shape?  It is
possible that endothelial SOCs are integral for
regulating pulmonary vascular permeability responses to
15  inflammatory mediators.  Whole lung studies (9, 18) have
shown that activation of SOCs alone is sufficient to
produce increased vascular permeability as assessed by
measures of the filtration coefficient.  In addition, SOC
activation promotes increased flux of macromolecules
20  across RPAEC monolayers (19, 26).  However, stimulation
of the thapsigargin-sensitive store-operated $Ca^{2+}$ entry
pathway in rat pulmonary microvascular endothelial cells
promotes neither increased macromolecular permeability
nor changes in cell shape (19).  These observations
25  suggest that inflammatory processes involving endothelial
SOC activation can produce pulmonary edema mediated by
the appearance of large-vessel leak sites away from the
gas-exchanging microcirculatory bed.  Therefore, future
studies are needed to determine whether 1) pulmonary
30  conduit-vessel endothelium and microvascular endothelium
represent distinct phenotypes having separate regulatory
properties, 2) changes in conduit-vessel endothelial cell
shape in situ lead to significant, function-comprising
pulmonary edema, 3) the precipitating factors for

increasing large-vessel and small-vessel (capillary)
permeabilities are the same, and 4) interventions to
alleviate pulmonary edema can be designed to selectively
target conduit-vessel endothelial cells vs. microvascular
5   endothelial cells.

The shape change elicited in response to SOC
activation in RPAECs has additional importance for other
endothelial-directed physiological processes such as
angiogenesis and regulation of leukocyte movement.  The
10  angiogenic process requires migration of endothelial
cells that, in turn, is dependent on the ability of cells
to change shape and decrease their cell-to-cell and cell-
to-matrix tethering (3).  Inhibition of non-voltage-gated
$Ca^{2+}$ channels, presumably including SOCs, inhibits
15  angiogenic factor-induced proliferation, migration, and
tube formation of human umbilical venous endothelial
cells (20), which are endothelial cells derived from
conduit vessels.  In addition, a study (17) has shown
that human umbilical venous endothelial cell-directed
20  regulation of leukocyte trafficking is $[Ca^{2+}]_i$ dependent.
Changes in endothelial cell shape and tethering that
accompany neutrophil adhesion and migration require
increased $[Ca^{2+}]_i$.  How the increased $[Ca^{2+}]_i$ occurs is not
clear, but a transmembrane $Ca^{2+}$ flux is required for
25  certain leukocyte secretory products to increase
endothelial $[Ca^{2+}]_i$ (32), thereby suggesting a role for
SOC-mediated $Ca^{2+}$ entry.  The data, in combination with
these findings, suggest that initiation sites for
angiogenesis and leukocyte diapedesis in vivo may be
30  located in pulmonary vascular segments lined with
endothelial cells possessing SOCs that regulate cell
shape.

In summary, the data shows that RPAECs possess
thapsigargin-activated SOCs that conduct current similar

to $I_{CRAC}$. RPAECs respond to this mode of $Ca^{2+}$ entry with changes in cell shape, interendothelial gap formation, and rearrangement of the F-actin cytoskeleton. Cytoskeletal rearrangement may be differentially

5 regulated by the extracellular and intracellular $Ca^{2+}$ pools, with $Ca^{2+}$ influx being necessary to produce a cytoskeleton configured for cell shape change. In addition, pulmonary endothelial cells from rats (and humans) express Trp1, which may be integral for forming

10 native SOCs in these cell types. Finally, pulmonary conduit vessel-derived endothelial SOC activation leading to interendothelial gap formation may be the basis for some forms of pulmonary edema and/or a component of angiogenesis and regulation of leukocyte trafficking to

15 and from the vasculature.

Although preferred embodiments have been depicted and described in detail herein, it will be apparent to those skilled in the relevant art that various

20 modifications, additions, substitutions and the like can be made without departing from the spirit of the invention and these are therefore considered to be within the scope of the invention as defined in the claims which follow.

Table A: sequence comparison (nucleotides 1-195) between
human Trp1 (hTrp1) and RT-PCR products from human PAECs
(HPAECs) and RPAECs. *Differences between RPAEC and hTrp1
sequences.

```
5              1
   hTrp1       TCG CCG AAC GAG GTG ATG GCG CTG AAG GAT
   HPAEC       TCG CCG AAC GAG GTG ATG GCG CTG AAG GAT
   RPAEC       TCG CCG AAC GAG GTG ATG GCG CTG AAG GAT


10 hTrp1       GTG CGG GAG GTG AAG GAG GAG AAT ACG CTG
   HPAEC       GTG CGG GAG GTG AAG GAG GAG AAT ACG CTG
   RPAEC       GTG CGA GAG GTG AAG GAG GAG AAC ACC TTG
                     *                        *   *   *


15 hTrp1       AAT GAG AAG CTT TTC TTG CTG GCG TGC GAC
   HPAEC       AAT GAG AAG CTT TTC TTG CTG GCG TGC GAC
   RPAEC       AAT GAG AAG CTT TTC TTG CTG GCG TGC GAC


   hTrp1       AAG GGT GAC TAT TAT ATG GTT AAA AAG ATT
20 HPAEC       AAG GGT GAC TAT TAT ATG GTT AAA AAG ATT
   RPAEC       AAG GGT GAC TAT TAT ATG GTT AAA AAG ATT


   hTrp1       TTG GAG GAA AAC AGT TCA GGT GAC TTG AAC
   HPAEC       TTG GAG GAA AAC AGT TCA GGT GAC TTG AAC
25 RPAEC       TTG GAG GAA AAC AGT TCA GGT GAC TTG AAC


   hTrp1       ATA AAT TGC GTA GAT GTG CTT GGG AGA AAT
   HPAEC       ATA AAT TGC GTA GAT GTG CTT GGG AGA AAT
   RPAEC       ATA AAT TGC GTA GAT GTG CTT GGG AGA AAT
30
   hTrp1       GCT GTT ACC ATA ACA     195
   HPAEC       GCT GTT ACC ATA ACA
   RPAEC       GCT GTT ACC ATA ACA
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Table B: deduced amino acid sequence (amino acids 27-91)
of RPAEC and HPAEC RT-PCR products with sequence
alignment.

```
                 27
   5  hTrpl    S P N E V M A L K D V R E V K E E N T L N E K L .
      HPAEC    S P N E V M A L K D V R E V K E E N T L N E K L .
      RPAEC    S P N E V M A L K D V R E V K E E N T L N E K L .


      hTrpl    - F L L A C D K G D Y Y M V K K I L E - E N S S G
  10  HPAEC    - F L L A C D K G D Y Y M V K K I L E - E N S S G
      RPAEC    - F L L A C D K G D Y Y M V K K I L E - E N S S G


      hTrpl    D L N I N C V D V L G R N A V T I T   91
      HPAEC    D L N I N C V D V L G R N A V T I T
  15  RPAEC    D L N I N C V D V L G R N A V T I T
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**What Is Claimed Is:**

1       1.     A method of decreasing inflammatory gaps in
2    pulmonary endothelial cells, the method comprising
3    decreasing levels of functional transient receptor
4    potential gene product in the cells.

1       2.     The method of claim 1 wherein decreasing levels
2    of functional transient receptor potential gene product
3    comprises decreasing transient receptor potential gene
4    expression in the cells.

1       3.     The method of claim 2 wherein decreasing
2    transient receptor potential gene expression comprises
3    exposing the cells to a compound which decreases
4    transient receptor potential gene expression.

·1       4.     The method of claim 3 wherein the compound is
2    an antisense oligonucleotide targeted to the transient
3    receptor potential gene.

1       5.     The method of claim 1 wherein decreasing levels
2    of functional transient receptor potential gene product
3    comprises exposing the cells to an inhibitor of the
4    functional transient receptor potential gene product.

1       6.     The method of claim 1 wherein decreasing levels
2    of functional transient receptor potential gene product
3    comprises exposing the cells to a compound which
4    interferes with membrane calcium channel formation by the
5    transient receptor potential gene product.

1      7.    A method of treating or preventing an
2  inflammatory condition in a subject, the method
3  comprising administering to the subject an amount of a
4  compound effective to decrease levels of functional
5  transient receptor potential gene product in the cells of
6  the subject.


1      8.    The method of claim 7 wherein the compound
2  decreases levels of functional transient receptor
3  potential gene product by decreasing transient receptor
4  potential gene expression.


1      9.    The method of claim 8 wherein decreasing
2  transient receptor potential gene expression comprises
3  exposing the cells to a compound which decreases
4  transient receptor potential gene expression.


1      10.   The method of claim 9 wherein the compound is
2  an antisense oligonucleotide targeted to the transient
3  receptor potential gene.


1      11.   The method of claim 7 wherein the compound is
2  an inhibitor of the functional transient receptor
3  potential gene product.


1      12.   The method of claim 7 wherein the compound
2  interferes with membrane calcium channel formation by the
3  transient receptor potential gene product.


1      13.   The method of claim 7 wherein the inflammatory
2  condition is asthma.

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FIG. 1A



FIG. 1B



FIG. 1C

FIG. 2A

NET INWARD CURRENT (pA/pF)

mV

● CONTROL
■ THAPSIGARGIN
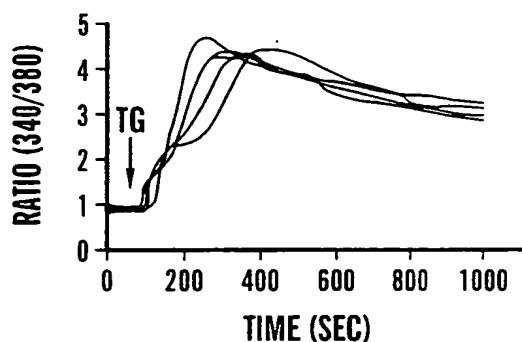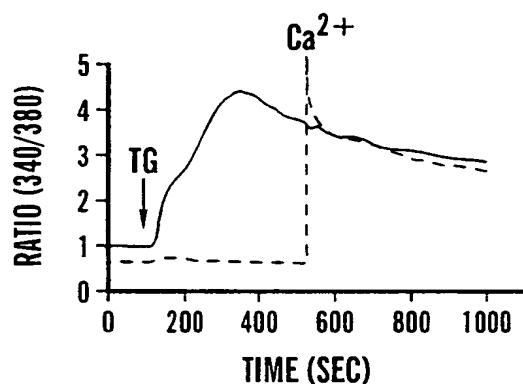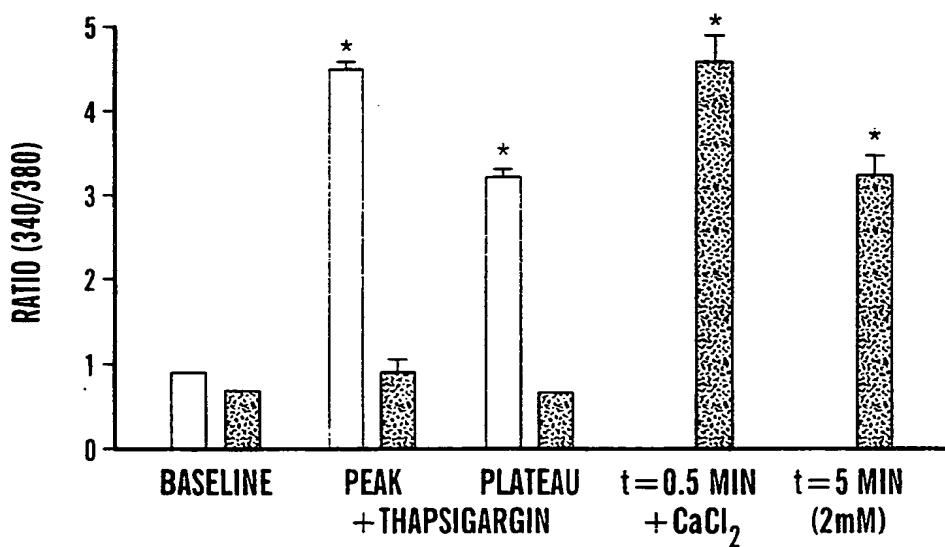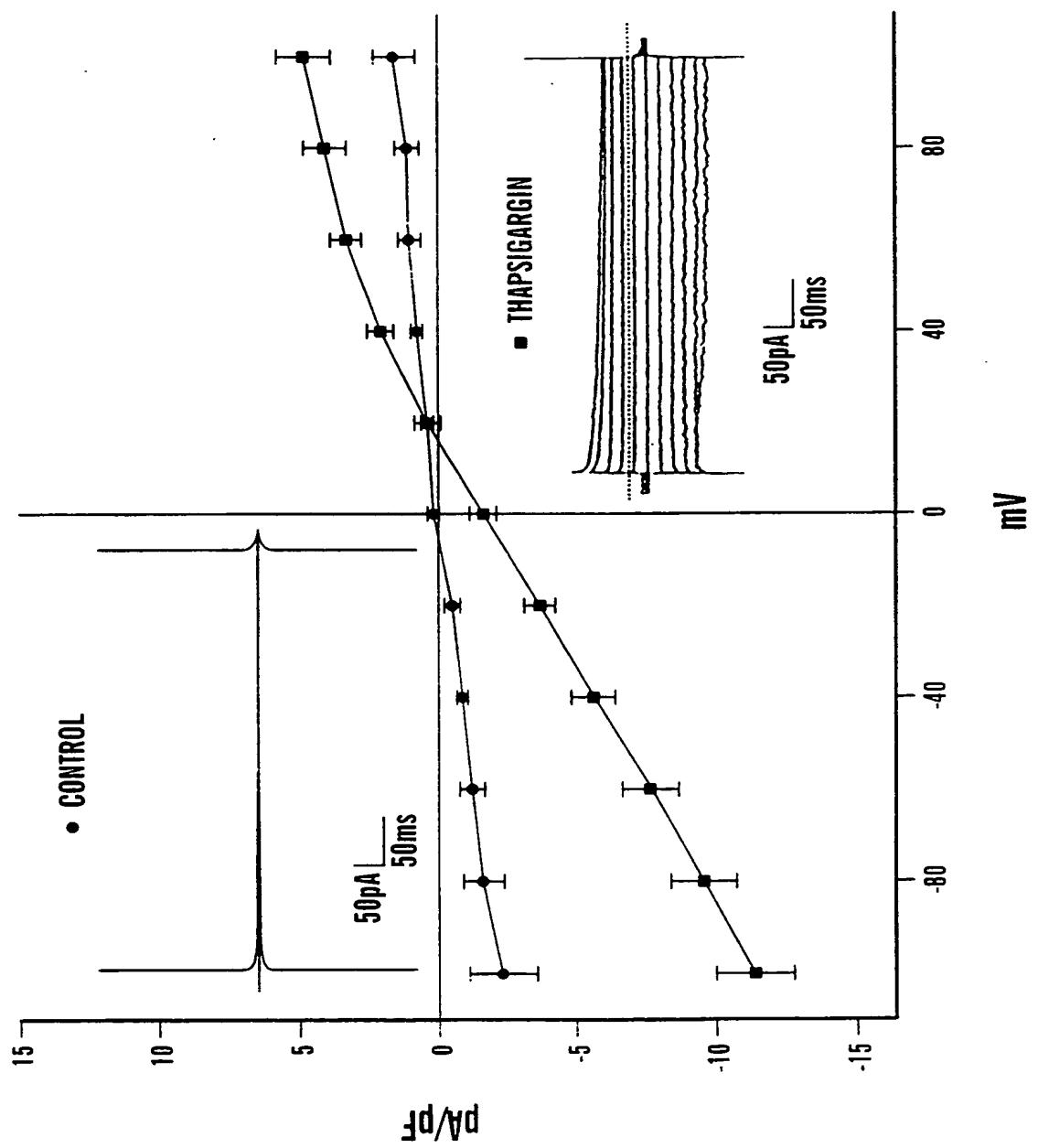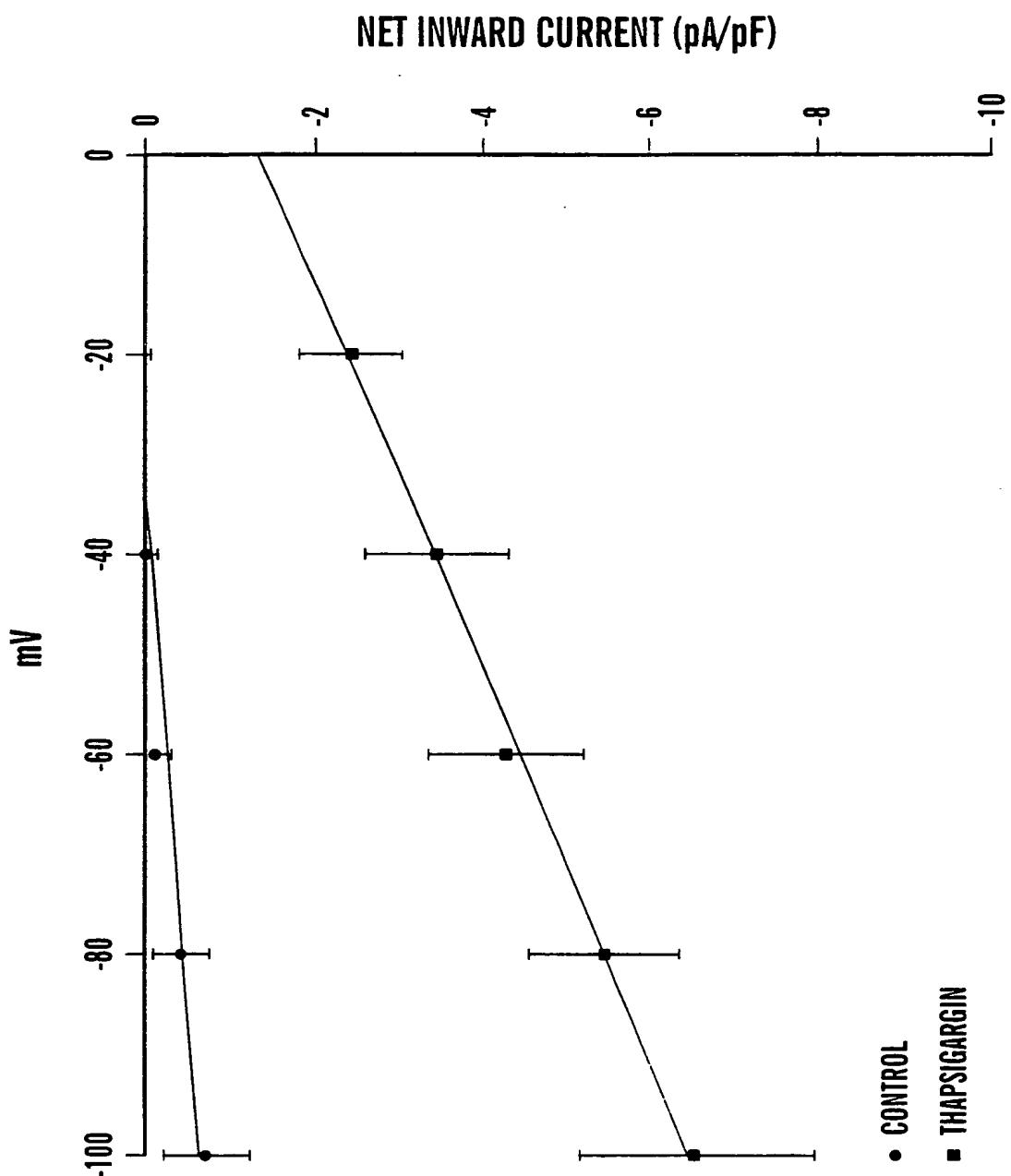
FIG. 2B

**FIG. 3A**



**FIG. 3B**



**FIG. 3C**



**FIG. 3D**

FIG. 4A



FIG. 4B
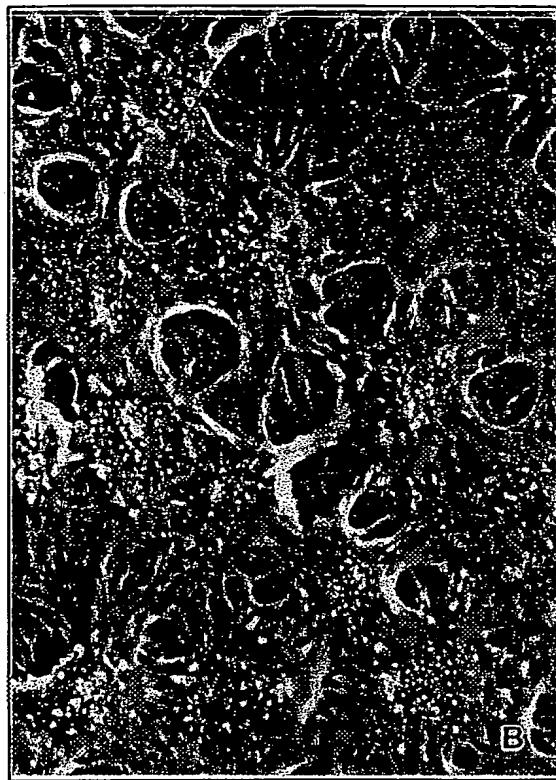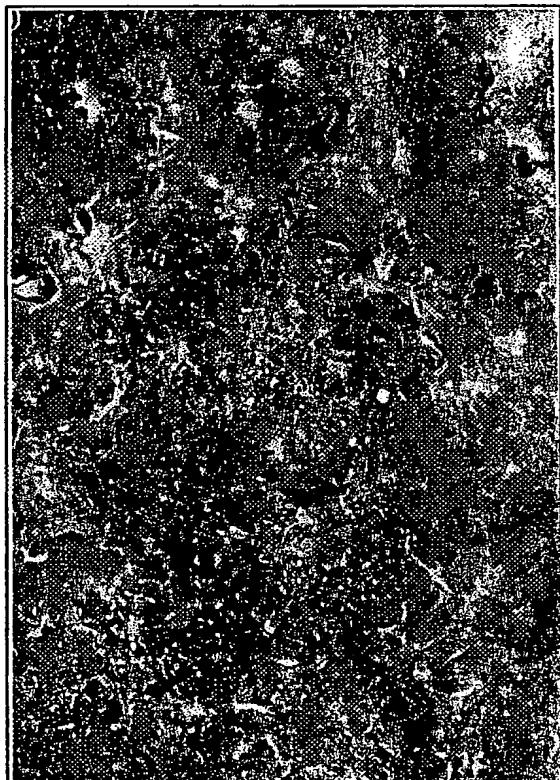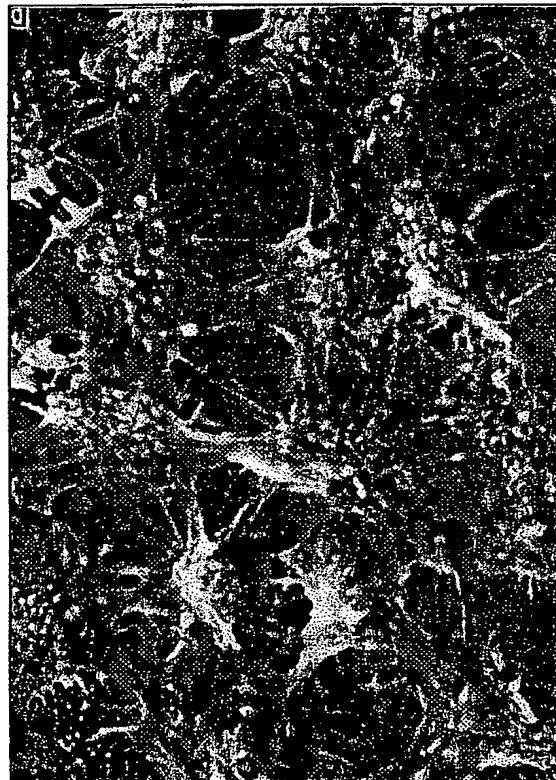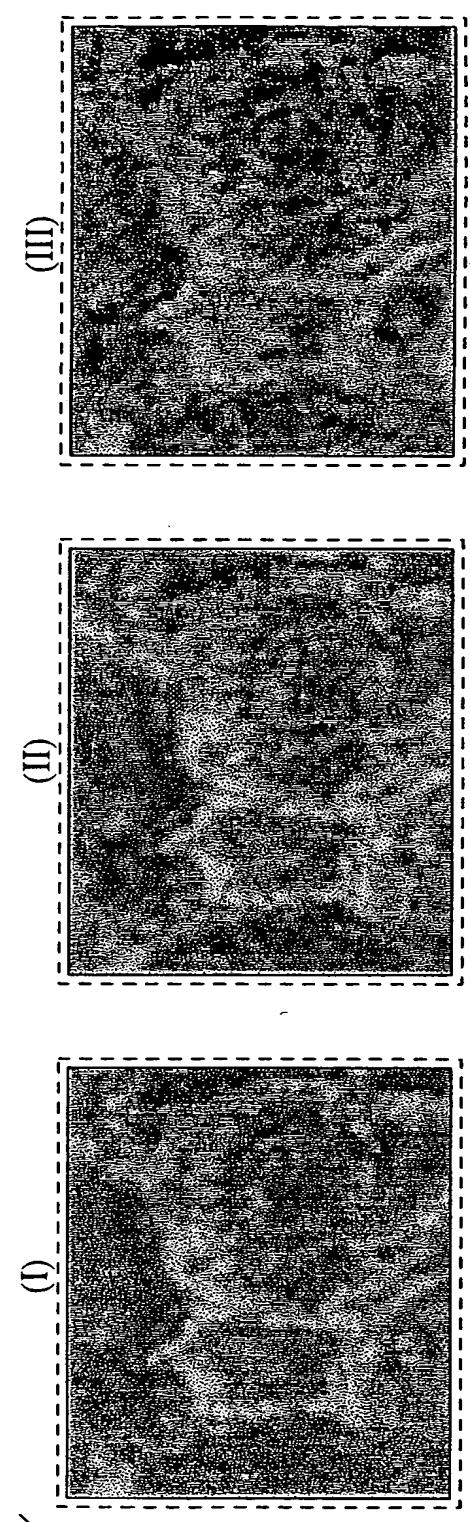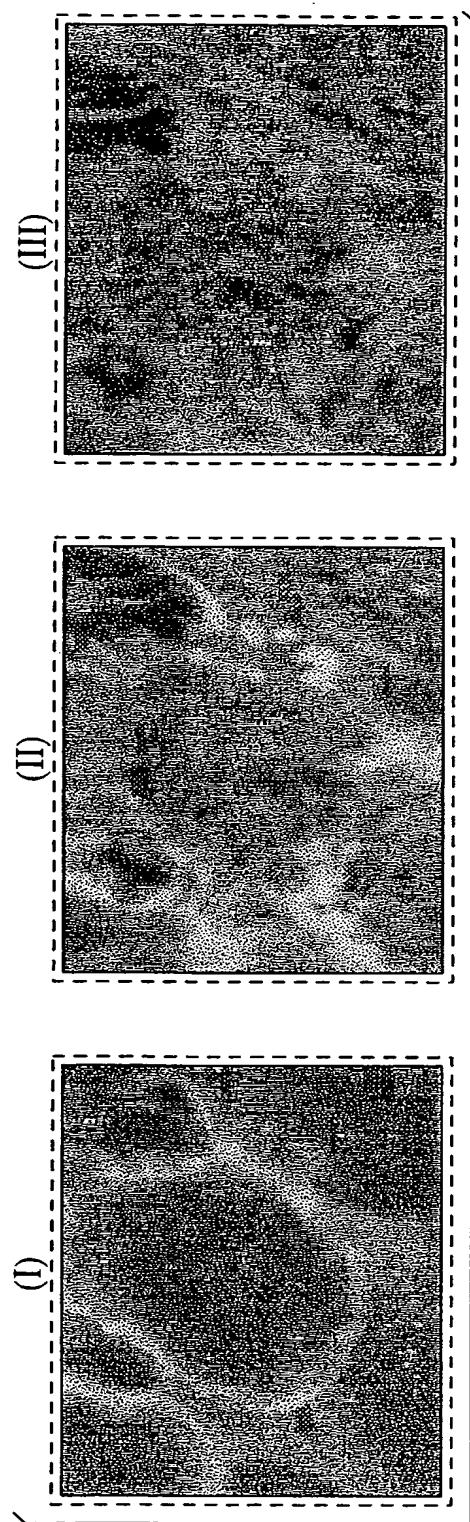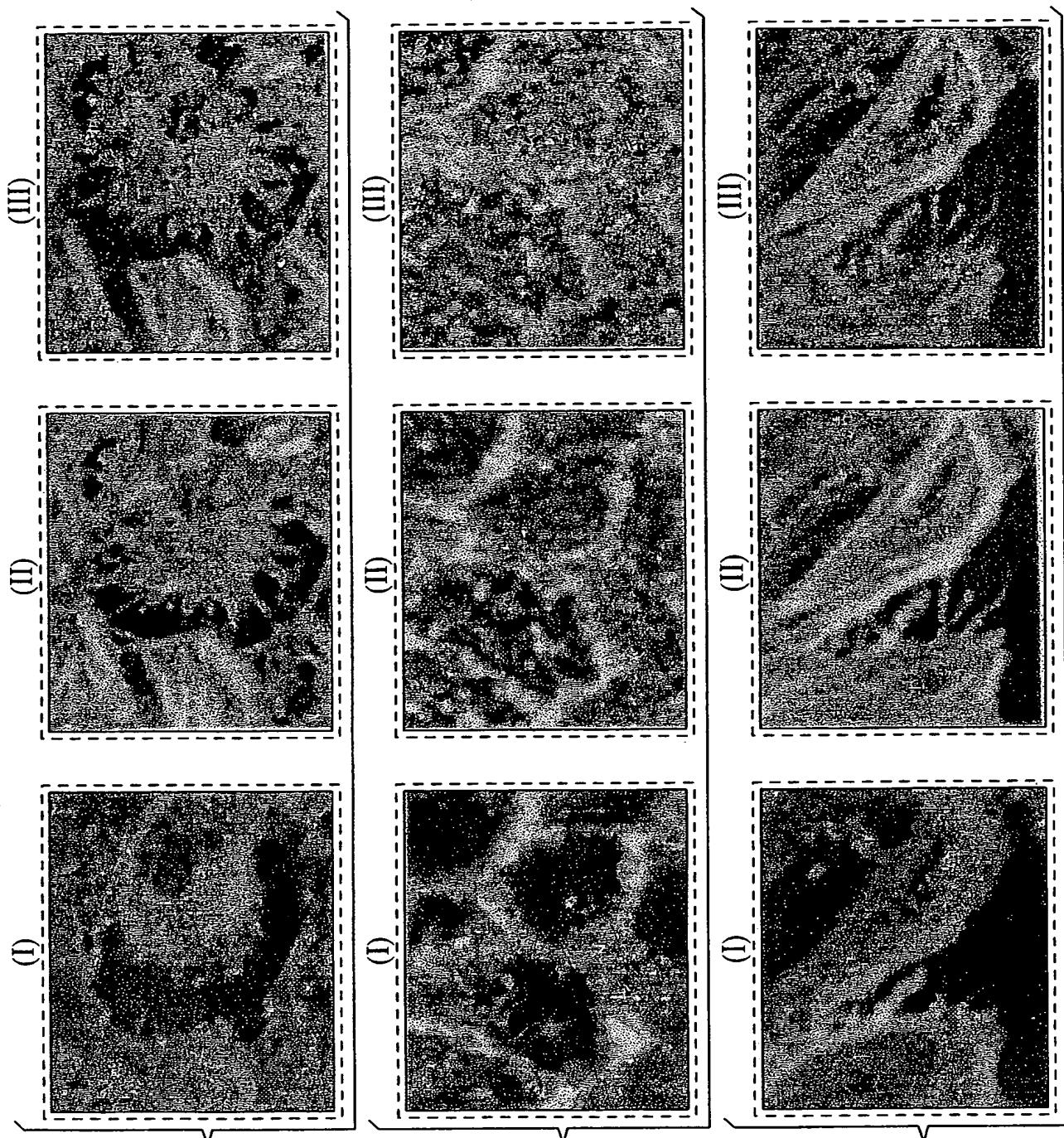
FIG. 5A

FIG. 5B

FIG. 5C

# INTERNATIONAL SEARCH REPORT

## A.   CLASSIFICATION OF SUBJECT MATTER

IPC(6)  : A61K 48/00; C12Q 1/68
US CL  : 435/6, 91.1, 375; 514/2, 44

According to International Patent Classification (IPC) or to both national classification and IPC

## B.   FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S.  :   435/6, 91.1, 375; 514/2, 44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS, EMBASE, CAPLUS, SCISEARCH

## C.   DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A, E | US 5,932,417 A (BIRNBAUMER et al) 03 August 1999, see entire document. | 1-13 |
| X | WO 98/08979 A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 05 March 1998, see entire document. | 1-13 |
| Y | TOMITA et al. Intracellular $Ca^{2+}$ Store-Operated Influx of $Ca^{2+}$ Through TRP-R, a Rat Homolog of TRP, Expressed in *Xenopus Oocytes*. Neuroscience Letters. 05 June 1998, Vol. 248, No. 3, pages 195-198, see entire document. | 1-13 |

☐  Further documents are listed in the continuation of Box C.      ☐      See patent family annex.

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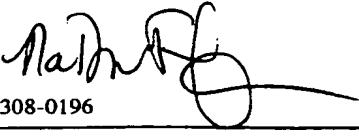
Form PCT/ISA/210 (second sheet)(July 1992)*